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A pilot study of the safety and efficacy of thymosin α_1 in augmenting immune reconstitution in HIV-infected patients with low CD4 counts taking highly active antiretroviral therapy

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SUMMARY

To study the safety and efficacy of thymosin α_1 in stimulating immune reconstitution in combination with highly active antiretroviral therarpy (HAART), a phase II randomized, controlled open-label trial of subcutaneous thymosin α_1 was undertaken for 12 weeks. Twenty clinically stable patients with viral loads <400 copies/ml and CD4 counts less than 200 cells/μl were randomized to receive 3·2 mg thymosin α_1 subcutaneous injections twice weekly or no injections for 12 weeks. CD4 and CD8 counts, CD45 RO^+ and RA^+ subsets and signal joint T cell receptor excision circles (sjTREC) in peripheral blood mononuclear cells (PBMCs) were measured every 2 weeks. Thirteen patients received thymosin α_1 and seven were controls. Thymosin α_1 was well tolerated and there were no serious adverse events. There was no significant difference between the thymosin α_1 and control groups in CD4, CD8 and CD45 lymphocyte subset changes at week 12; however, PBMC sjTREC levels increased significantly in the thymosin α_1-treated patients compared to controls at week 12. In conclusion, the increase in PBMC sjTREC levels in patients taking thymosin α_1 may represent enhanced immune reconstitution; however, the clinical benefits and long-term consequences remain to be determined.

Keywords CD4 HIV, immune reconstitution thymopoiesis thymosin α_1

INTRODUCTION

Treatment of HIV-infected patients with highly active antiretroviral therapy (HAART) leads to immune reconstitution as shown by increases in CD4 lymphocyte counts, decreased risk of opportunistic infections and improved survival [1,2]. A minority of patients however, especially those starting therapy with low CD4 counts, have slow and incomplete recovery of CD4 cells and such individuals appear to have a greater risk of AIDS-associated events than patients who experience more substantial recovery of CD4 counts [3,4]. These patients may benefit from the adjunctive use of immunomodulatory compounds that enhance immune reconstitution.

Thymosin α_1 (thymalfasin) is a 28-amino acid thymic peptide that is homologous to a natural product originally isolated from thymosin fraction 5 of calf thymuses [5]. It has various immunomodulatory properties that lead to augmentation of T lymphocyte function, including modulation of interleukin-2 (IL-2) [6,7], stimulation of interferon-γ (IFN-γ) production [8], induction of T lymphocyte and natural killer (NK) cells [9,10] and stimulation of thymopoiesis [11–14]. Thymosin α_1 has also been shown to up-regulate MHC Class I expression in antigen-presenting cells [15].

In previous clinical trials in HIV-infected patients, thymosin α_1 was shown to induce increases in CD4 counts, but only when given in combination with either interleukin (IL)-2 or interferon (IFN)-α [16–18]. However, these studies were conducted in patients taking zidovudine monotherapy and the efficacy of thymosin α_1 in patients with full viral suppression on combination antiretroviral therapy is unknown. The earlier studies showed that the drug is very well tolerated, which makes it potentially more attractive as an immune stimulant than IL-2 or IFN-α as these are associated with substantial toxicity.

We therefore conducted a pilot study to determine the safety and efficacy of thymosin α_1 in patients taking HAART. In addition to the primary outcome of increase in CD4 lymphocytes, we also wished to investigate the potential of thymosin α_1 to increase *de novo* naive T cell production as this has been postulated to be an important component of immune reconstitution [19,20].

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MATERIALS AND METHODS

Subjects

Twenty HIV-infected outpatients attending the Communicable Diseases Centre, Tan Tock Seng Hospital, Singapore were enrolled between December 2000 and April 2001. The inclusion criteria were confirmed HIV infection (positive ELISA and Western blot); age greater than 18 years; current use of combination antiretroviral therapy with no change or interruption in regimen in the previous 4 months; undetectable viral load (less than 400 copies/ml) and CD4 count below 200 cells/μl at the time of study entry. The exclusion criteria included current treatment for active opportunistic infections; presence of hepatitis B surface antigen or hepatitis C antibodies; concomitant use of corticosteroids, hydroxyurea or other immunomodulatory agents; abnormal haematological (haemoglobin <8 g/dl, platelet count $<50 \times 10^9$/l or absolute neutrophil count $<0{\cdot}75 \times 10^9$/l) or biochemical (serum creatinine >2 mg/dl, alanine transaminase, alkaline phosphatase or amylase >five times the laboratory's upper limit of normal) profile.

The study was approved by the Ethics Committee of Tan Tock Seng Hospital and all subjects gave written informed consent prior to participation in the study.

Study design

Patients were randomized in the ratio of 3 : 2 to thymosin or control group. Randomization was performed using opaque envelopes containing study codes that had been preprepared by a member of research staff not directly involved in the study.

Study treatment

Patients randomized to the thymosin group received 6·4 mg thymosin α_1 weekly by subcutaneous injection in two divided doses. This dose was chosen as it was at the upper range of that used previously, and well tolerated, in studies on HIV-infected subjects [16–18]. Thymosin α_1 (Thymalfasin; Sci Clone Pharmaceuticals Inc., San Mateo, CA, USA) was supplied as a lyophilized powder in vials containing 1·6 mg of thymosin 1α, 50 mg mannitol and sodium phoshate buffer to adjust the pH to 6·8. The powder was reconstituted with 1 ml of sterile water for injection. Patients randomized to the control group received no injections during the period of the study, but were offered the chance of receiving thymosin after completion of the 12-week study period. All patients were instructed to continue their usual antiretroviral therapy throughout the study period.

Assessments

Safety evaluation and CD4 response. All subjects had a clinical evaluation at baseline, weeks 4, 8 and 12 of the study. Each evaluation included a review of symptoms, physical examination and measurement of a full blood count and biochemical safety parameters (electrolytes, liver function tests, glucose and amylase). Clinical and laboratory adverse events were graded using standard toxicity criteria.

Plasma viral load was measured at baseline and week 12 using the Amplicor Ultrasensitive test (Roche, Nutley, NJ, USA). CD4 and CD8 counts were measured at each study visit by flow cytometry and performed blinded to the subject's treatment allocation. Blood was also collected at each study visit for extraction of peripheral blood mononuclear cells (PBMC). These were cryopreserved at the study site and immunological assays were performed later on the batch of samples.

Naive and memory lymphocyte subset and sjTREC analysis. Immunological assays on the frozen PBMCs were performed at the Department of Immunology, Imperial College of Science, Technology and Medicine, London, UK. Detailed phenotypic evaluation was carried out using four-colour flow cytometry on a Beckton Dickinson FACScalibur™ flow cytometer. Quantification of cell-surface receptor expression on $CD4^+$ and $CD8^+$ T cells was performed on 10^5 cryopreserved PBMC by six-parameter flow cytometry using commercially available antibodies (Beckman Coulter). Cells were labelled with a cocktail of murine MoAbs: allophycocyanin (APC)-conjugated antihuman CD8, phycocyanin 5 (PC5)-conjugated antihuman CD4, fluorescein isothiocyanate (FITC)-conjugated antihuman CD45RA, phycoerythrin (PE)-conjugated antihuman CD45RO, FITC-conjugated antihuman HLA-DR, PE-conjugated antihuman CD25 and PE-conjugated anti-Ki67, for 30 min at 4°C. After staining, cell suspensions were washed once in phosphate buffered saline (PBS) and fixed in 300 μl 2% paraformaldehyde solution in PBS. For acquisition, a gate was set around the lymphocyte population on a forward scatter *versus* a side-scatter dot plot, and 10 000 gated events were collated for each sample. Data analysis was performed using CELLQuest™ software. Appropriate isotype-matched controls were run in parallel for each sample.

Signal joint T cell receptor rearrangement excision circles (sjTREC) analysis was performed on cryopreserved PBMCs, from which DNA was extracted (from 4×10^6 cells) using the Puregene DNA purification kit (Gentra, Flowgen, Staffordshire, UK). PCR amplification of sjTREC was performed according to the method described by Douek *et al*. [21]. PCR products were run on 1% agarose gels containing 0·0005% ethidium bromide and visualized using a transilluminator. Quantification of sjTREC numbers was performed using a standard curve according to the method previously described [22].

Statistical analysis

The primary end-point was the change in CD4 count from baseline to week 12. A target of 20 patients was estimated to be adequate to detect a difference in CD4 count of 100 cells/μl between the groups with 80% power at a significance level of less than 5%.

Secondary outcome parameters were change in CD8 count at week 12, change in CD4 count at weeks 4 and 8, change in CD45 RO^+ and RA^+ in CD8 and CD4 cells at weeks 4, 8 and 12, and change in PBMC sjTREC at weeks 4, 8 and 12. Changes in lymphocyte subsets and sjTREC were analysed using ANOVA or the Mann–Witney *U*-test according the distribution of the data. Data were analysed with SPSS (version 9·0) software.

RESULTS

Characteristics of subjects

The characteristics of the study subjects at baseline are shown in Table 1. Twenty subjects were enrolled, most of whom were men of Chinese race. HAART regimens were varied, but were generally similar between the two groups, with four of the seven controls (57%) and six of the 13 thymosin patients (46%) taking protease-inhibitor based regimens. There were no significant differences between patient characteristics in each group. All patients completed the protocol and were evaluable for safety and efficacy at week 12.

Table 1. Demographic features and baseline parameters of patients in the thymosin and control groups

Characteristic	Control group (n = 7)	Thymosin group (n = 13)
Age in years, mean (range)	42 (31–61)	47 (33–62)
Years since HIV diagnosis, mean (range)	2·29 (1–4)	2·15 (1–6)
HIV disease stage, number (%)		
B	1 (14)	3 (23)
C	6 (86)	10 (77)
Antiretroviral therapy, number (%)		
2NRTI + PI	2 (29)	5 (38)
2NRTI + NNRTI	1 (13)	5 (38)
3 NRTI	2 (29)	1 (8)
Other combination	2 (29)	2 (16)
Duration of antiretroviral therapy in months, mean (range)	13 (6–19)	13 (3–44)
HIV viral load, number (%)		
<50 copies/ml	5 (71)	10 (77)
50–400 copies/ml	2 (29)	3 (23)
CD4 cells/ml, median (range)	94 (66–171)	102 (36–145)
CD8 cells/ml, median (range)	882 (450–1192)	920 (350–1022)

Table 2. Change in mean lymphocyte subset numbers (cells/μl) and sjTREC (copies/μl blood) ± s.d. from baseline over the study in the thymosin and control groups. P-value denotes significance as calculated by the unpaired t-test

	Week 4			Week 8			Week 12		
	Thymosin	Control	P	Thymosin	Control	P	Thymosin	Control	P
Total CD4	12 ± 27	26 ± 39	0·41	51 ± 59	43 ± 43	0·74	30 ± 30	51 ± 38	0·18
Total CD8	119 ± 24	– 29 ± 339	0·25	257 ± 305	55 ± 165	0·15	187 ± 150	225 ± 244	0·67
CD45 RA + CD4+	12 ± 17	19 ± 16	0·39	28 ± 29	18 ± 14	0·51	– 0·3 ± 17	10 ± 15	0·22
CD45 RA + CD8+	– 86 ± 113	– 115 ± 169	0·68	– 52 ± 151	13 ± 188	0·48	– 11 ± 155	48 ± 172	0·47
CD45 RO + CD4+	1 ± 45	8 ± 23	0·71	28 ± 45	30 ± 33	0·91	38 ± 30	32 ± 61	0·77
CD45 RA + CD8+	82 ± 225	– 64 ± 349	0·29	160 ± 314	– 38 ± 138	0·21	163 ± 107	164 ± 270	1·00
sjTREC	3·5 ± 11·1	5 ± 7·2	0·60	– 1·3 ± 8·1	6·3 ± 7·2	0·83	27·9 ± 48·3	– 3·9 ± 7·2	0·035

Safety and tolerability

All patients randomized to thymosin α_1 injections completed the 12 weeks of treatment, with none requiring dose reduction or discontinuation. One patient in the control group had a Grade 3 elevation of serum amylase at week 12, but this was not accompanied by symptoms and it resolved spontaneously. All other adverse events were of Grade I severity, and there was no significant difference in frequency of adverse events between the two groups. Viral load increased to detectable levels (> 400 copies/ml) in three patients at week 12.

Lymphocyte subsets

Changes in total CD4+ and CD8+ lymphocyte counts over the 12 weeks are shown in Table 2. CD4 and CD8 lymphocyte counts showed upward trends in both groups over the study period but there was no significant difference between the thymosin and control group in either parameter. There were no differences seen between the two groups in either absolute numbers or percentage of memory or naive CD4 or CD8 T cells over the study period (Table 2).

SjTREC evaluation

There was no significant difference in Ki67 levels between the two groups at any stage, suggesting differences in lymphocyte proliferation. The mean level of sjTREC was similar in the two groups at baseline (5·7 *versus* 5·5 copies/μl; P = 0·98). Changes in sjTREC over the study period are shown in Table 2 and Fig. 1. There was an increase in mean sjTREC levels in the thymosin group at week 12 that was significant in comparison to controls (+27·9 compared with –3·9 copies/μl; P = 0·035).

DISCUSSION

This pilot study was conducted to determine the safety and efficacy of thymosin α_1 injections in conjunction with combination antiretroviral therapy in patients with advanced HIV infection. We demonstrated that thymosin α_1 was very well tolerated in this group of patients, which is in agreement with other clinical trials of thymosin α_1 in HIV-infected patients and in patients with hepatitis B and C infection [16–18,23]. The good tolerability contrasts with immunomodulatory cytokines such as IFN-α or IL-2 that

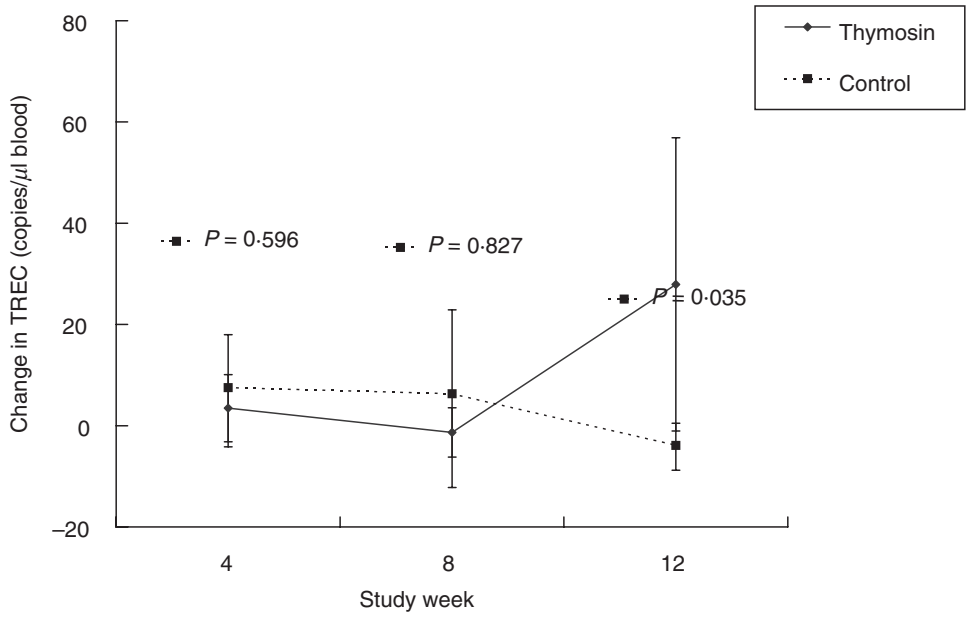

Fig. 1. Change in sjTREC levels (copies/μ1 blood) in PBMCs from study patients. Error bars denote 95% confidence intervals and *P* value calculated using the Mann–Witney *U*-test.

have been shown to have substantial toxicity in patients with HIV infection as well as other groups.

We did not find any benefit of thymosin α_1 in augmenting CD4 count response to HAART. Although the study was small and therefore had statistical power only to detect a relatively large difference in CD4 cells, the lack of any trend towards higher values in the thymosin group suggests that the drug is not effective in inducing a substantial rise in CD4 counts at a dose of 6·4 mg weekly given for 12 weeks. It is conceivable that a higher dose of thymosin α_1 might have a greater effect on CD4 cells, although the dose that we chose for the study was relatively high in comparison to previous studies in patients HIV infection and currently recommended dosage for use in the treatment of hepatitis virus infections. It is also possible that an effect might have become apparent after a longer duration of therapy. In one previous trial, thymosin (given in conjunction with or IFN-α) had its greatest effect after 12 months of treatment [17]. We selected a group of patients with advanced immunodeficiency for this study, and it is also possible that a greater CD4 response might be obtained in patients with higher initial CD4 counts.

Immunophenotyping studies were performed to assess the effect of thymosin 1α in stimulating expansion of naive and memory T cells. HAART has been shown to allow reconstitution of both subsets of T cells [20,21,24], and there is recent evidence that one mechanism for this effect is through production of *de novo* naive T lymphocytes in the thymus [20,25]. Some studies have suggested that impaired T cell restoration in patients with low CD4 counts when starting HAART may result from impaired thymic function [24]. The lack of any specific effect of thymosin 1α in this study, in terms of increases in numbers of naive or memory $CD4^+$ or $CD8^+$ T cells, did not support a role of thymosin in selectively stimulating these particular lymphocyte subtypes.

The most interesting effect of thymosin was the significant increase in sjTREC levels seen after 12 weeks of treatment. sjTREC is a technique for measuring *de novo* T cell production, and sjTREC levels have been shown to correlate with recent thymic emigration and thus thymopoiesis [21,26]. Increase in the *de novo* production of naive T cells by thymopoiesis may be an important component of immune reconstitution in HIV-infected patients taking HAART [19,20], and sjTREC levels have been shown to predict survival in HIV-infected patients [27] as well as the total CD4 count response to HAART after several years [24,28,29]. Although we measured levels of sjTREC in PBMCs in this study (rather than in specific lymphocyte subpopulations), it is likely that the increase in TREC in the thymosin 1α group at week 12 reflects an increase in naive lymphocytes which are recent thymic emigrants. However, the expansion of *de novo* naive T cells is likely to be small, given that the total numbers of T cells were not higher in the subjects receiving thymosin 1α. Furthermore, we did not observe an increase in the naive T cell population as determined by immunophenotype. A possible explanation for this discrepancy might be the rapid acquisition of a memory phenotype by de novo naïve cells in the presence of a lymphopenic environment, as has been observed previously [30,31]. It might be the case that such *de novo* T cells, regardless of phenotype, are functionally important in recovery of immune responses. Further studies are needed to confirm and further clarify the immunological changes and to determine the clinical significance, if any, of these findings.

In summary, we have shown that a 12-week course of thymosin 1α increased levels of sjTREC but not CD4 counts in a group of patients with advanced HIV disease receiving HAART. Thymosin 1α appeared to be very well tolerated and its potential for clinical use to augment the immune response to HAART may warrant further investigation in studies of longer treatment duration.

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